(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 585 660 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
03.05.2000 Bulletin 2000/18

(21) Application number: **93112737.7**

(22) Date of filing: **09.08.1993**

(51) Int. Cl.[7]: **C12Q 1/68**, C12Q 1/44, C12N 9/12, C12N 9/16, C12P 19/34

(54) Exonuclease decontamination method

Exonuklease-Dekontaminierungsverfahren

Procédé de décontamination utilisant d'exonuclease

(84) Designated Contracting States:
DE FR GB IT

(30) Priority: **17.08.1992 US 931241**

(43) Date of publication of application:
09.03.1994 Bulletin 1994/10

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(56) References cited:
WO-A-92/00384

- **NUCLEIC ACIDS RESEARCH. vol. 19, no. 9 , May 1991 , ARLINGTON, VIRGINIA US page 2511 ZHU, Y ET AL 'The use of exonuclease III for polymerase reaction sterilization'**
- **NUCLEIC ACIDS RESEARCH. vol. 19, no. 11 , June 1991 , ARLINGTON, VIRGINIA US pages 3139 - 3141 LI, H. ET AL. 'Eliminating primers from completed polymarase chain reactions with exonuclease VII'**

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Printed by Xerox (UK) Business Services
2.16.7 (HRS)/3.6

the method of the present invention may be carried out by any suitable amplification reaction as described above, with SDA again preferred.

[0011] Strand displacement amplification (SDA) may be carried out in accordance with known techniques. See generally G. Walker et al., Proc. Natl. Acad. Sci. USA 89, 392-396 (1992); G. Walker et al., Nucleic Acids Res. 20, 1691-1696 (1992). For example, SDA may be carried out with a single amplification primer or a pair of amplification primers, with exponential amplification being achieved with the latter. In general, SDA amplification primers comprise, in the 5' to 3' direction, a flanking sequence (the DNA sequence of which is noncritical), a restriction site for the restriction enzyme employed in the reaction, and an oligonucleotide sequence (e.g., an oligonucleotide probe of the present invention) which hybridizes to the target sequence to be amplified and/or detected. The flanking sequence, which serves to facilitate binding of the restriction enzyme to the recognition site and provides a DNA polymerase priming site after the restriction site has been nicked, is preferably about 15 to 20 nucleotides in length; the restriction site is functional in the SDA reaction (i.e., phosphorothioate linkages incorporated into the primer strand do not inhibit subsequent nicking--a condition which may be satisfied through use of a nonpalindromic recognition site); the oligonucleotide probe portion is preferably about 13 to 15 nucleotides in length.

[0012] SDA is carried out with a single amplification primer as follows: a restriction fragment (preferably about 50 to 100 nucleotides in length and preferably of low GC content) containing the sequence to be detected is prepared by digesting a DNA sample with one or more restriction enzymes, the SDA amplification primer is added to a reaction mixture containing the restriction fragment so that a duplex between the restriction fragment and the amplification primer is formed with a 5' overhang at each end, a restriction enzyme which binds to the restriction site on the amplification probe (e.g., HincII) is added to the reaction mixture, an exonuclease deficient DNA polymerase (e.g., an exonuclease deficient form of E. coli DNA polymerase I, see V. Derbyshire, Science 240, 199-201 (1988)) is added to the reaction mixture, and three dNTPs and one dNTP[∝S], with the dNTP[∝S] selected so that a phosphorothioate linkage is incorporated into the primer strand at the restriction site for the particular restriction enzyme employed (e.g., dGTP, dCTP, dTTP, and dATP[∝S] when the restriction enzyme is HincII) are added to the reaction mixture. The DNA polymerase extends the 3' ends of the duplex with the dNTPs to form a downstream complement of the target strand, the restriction enzyme nicks the restriction site on the amplification primer, and the DNA polymerase extends the 3' end of the amplification primer at the nick to displace the previously formed downstream complement of the target strand. The process is inherently repetitive because the restriction enzyme continuously nicks new complementary strands as they are formed from the restriction site, and the DNA polymerase continuously forms new complementary strands from the nicked restriction site.

[0013] SDA can also be carried out with a pair of primers on a double stranded target DNA sequence, with the second primer binding to the 3' end of the complementary strand, so that two sets of repetitive reactions are occurring simultaneously, with the process proceeding exponentially because the products of one set of reactions serve as a target for the amplification primer in the other set of reactions.

[0014] The step of first digesting the DNA sample to form a restriction fragment in an SDA reaction can be eliminated by exploiting the strand displacing activity of the DNA polymerase and adding a pair of "bumper" primers which bind to the substrate at a flanking position 5' to the position at which each amplification primer binds. Each bumper primer extension product displaces the corresponding amplification primer extension product. Amplification primers, which are present in excess, then bind to the displaced primer extension products, and upon extension, a double-stranded DNA fragment is formed which can then serve as a substrate for exponential SDA with that pair of amplification primers.

[0015] Polymerase chain reaction (PCR) may also be carried out in accordance with known techniques. See, e.g., U.S. Patents Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosure of all U.S. Patent references cited herein are to be incorporated herein by reference). In general, PCR involves, first, treating a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) with one oligonucleotide primer for each strand of the specific sequence to be detected under hybridizing conditions so that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith so that the extension product synthesized from each primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and then treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present. These steps are cyclically until the desired degree of amplification is obtained. Detection of the amplified sequence may be carried out by adding to the reaction product an oligonucleotide probe capable of hybridizing to the reaction product (e.g., an oligonucleotide probe of the present invention), the probe carrying a detectable label, and then detecting the label in accordance with known techniques.

[0016] Ligase chain reaction (LCR) is also carried out in accordance with known techniques. See, e.g., R. Weiss, Science 254, 1292 (1991). In general, the reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. Each pair together completely overlaps the strand to which it corresponds. The reaction is carried out by, first, denatur-

(e.g., pH, salt and temperature), and (5) the relative lengths of the contaminating amplicon and the fragment that contains the target sequence. Each system can routinely be empirically optimized by varying the above variables and determining those conditions which most efficiently destroy contaminating amplicons while retaining sufficient levels of target sequence for subsequent amplification.

[0023] The present invention is explained in greater detail in the following Examples. In the Examples, µg means micrograms, µL means microliters, mL means milliliters, mM means millimolar, nM means nanomolar, v/v means volume/volume, cpm means counts per minute, and temperatures are given in degrees Centigrade unless otherwise indicated. These Examples are for illustrative purposes only, and are not to be taken as limiting of the invention. All samples in the following examples contained human placental DNA and/or calf thymus DNA in an attempt to mimic clinical M. tb samples which tend to contain human DNA.

EXAMPLE 1

Amplicon Products from a Previous Strand Displacement Amplification (SDA) Reaction

[0024] An SDA reaction was performed in accordance with known techniques, see G. Walker et al., Nucleic Acids Res. 20, 1691-1696 (1992), using genomic target DNA from Mycobacterium tuberculosis (M. tb) prepared in accordance with known techniques. See S. Visuvanathan et al., J Microbiol. Methods 10, 59 (1989). A 50 µL sample containing the following reagents was assembled: 50 mM K_2PO_4, pH 7.6; 100 µg/mL bovine serum albumin; 1 mM each dGTP, dCTP, TTP dATPαS; 3% (v/v) 1-methyl 2-pyrrolidinone 6 mM $MgCl_2$; 5000 genome copies of DNA from M. tb; 0.25 µg of human placental DNA; primer #1 to 500 nM (SEQ ID NO:1)
primer #2 to 500 nM (SEQ ID NO:2); primer #3 to 50 nM (SEQ ID NO:3); and primer #4 to 50 nM (SEQ ID NO:4).

[0025] The sample was heated 2 minutes at 95° C followed by 2 minutes at 37° C. SDA proceeded 2 hours at 37° C after addition of 5 units of exo- klenow and 150 units of HincII. Genomic target DNA from M. tb DNA was amplified to a level of 10^{10} amplicons µL.

EXAMPLE 2

Decontamination of Amplicons with Exonuclease I for 22 Minutes

[0026] An array of samples containing various amounts of either genomic target DNA from Mycobacterium tuberculosis (M. tb) or amplicons (prepared in Example 1) were assembled as indicated in Table 1. Each 50 µL sample contained the following reagents: 50 mM K_2PO_4, pH 7.6
100 µg/mL bovine serum albumin; 1 mM each dGTP, dCTP, TTP dATPαS; 3% (v/v) 1-methyl 2-pyrrolidinone; 6 mM $MgCl_2$
0.5 µg of human placental DNA; 0 or 18 µg of calf thymus DNA (Table 1); and M. tb genomic DNA or amplicons (Table 1).

[0027] Samples were heated 2 minutes at 95° C followed by 2 minutes at 37° C. Exonuclease I was added to samples as indicated in Table 1 followed by incubation for 22 minutes at 37° C. Exonuclease I was inactivated by heating 10 minutes at 56° C. SDA proceeded 2 hours at 37° C upon addition of: primer #1 to 500 nM (SEQ ID NO:1); primer #2 to 500 nM (SEQ ID NO:2); primer #3 to 50 nM (SEQ ID NO:3)
primer #4 to 50 nM (SEQ ID NO:4); 5 units of exo-klenow, and 150 units of HincII.

[0028] SDA products were detected by hybridization and extension of an amplicon specific ^{32}P-probe (SEQ ID NO: 5) followed by analysis using denaturing gel electrophoresis and quantitation of SDA products (^{32}P cpm). See Walker et al., supra.

[0029] In the presence of 0.5 µg of human DNA, a sample containing 1000 added amplicons produces a signal of 3030000 cpm after strand displacement amplification (Table 1). Prior treatment of an identical sample with 15 units of exonuclease I for 22 minutes results in a signal of 28700 cpm which is essentially equal to the background signal obtained for an exonuclease treated sample lacking added amplicons (25300 cpm).

TABLE 1

Decontamination For 22 minutes with Exonuclease I				
amplicons (molecules)	M.tb target DNA (genome molecules)	Exonuclease I (units)	human/calf thymus DNA (µg)	SDA product (^{32}P cpm)
0	10	0	0.5	184000

exo- klenow; and 150 units of HincII.

[0033] SDA products were detected by hybridization and extension of an amplicon specific ^{32}P-probe (SEQ ID NO:5) followed by analysis using denaturing gel electrophoresis and quantitation of SDA products (^{32}P cpm) (Walker et al., supra.).

TABLE 2

Decontamination For 30 minutes with Exonuclease I				
amplicons (molecules)	M.tb target DNA (genome molecules)	Exonuclease I (units)	human DNA (µg)	SDA product (^{32}P cpm)
0	10^3	0	0.5	700000
0	0	0	0.5	35800
0	10^3	10	0.5	509000
0	0	10	0.5	20200
0	10^3	25	0.5	1000000
0	0	25	0.5	31700
0	10^3	10	15	165000
0	0	10	15	2200
0	10^3	25	15	280000
0	0	25	15	4800
10^5	0	0	0.5	1310000
10^5	0	10	0.5	103000
10^5	0	25	0.5	104000
10^6	0	25	0.5	675000
10^5	0	10	15	11400
10^5	0	25	15	24400

[0034] In the presence of 0.5 or 15 µg of human DNA, treatment with either 10 or 25 units of exonuclease I for 30 minutes reduces amplification of 10^5 amplicons to a level about 5 fold over background (Table 2). For example, the SDA signal from 10^5 amplicons is reduced from 1310000 to 103000 cpm upon prior treatment with exonuclease I. A signal of 103000 cpm is about 5 fold greater than the corresponding background signal from a sample with no added amplicons (20200 cpm). Amplification of genomic M. tb DNA is not significantly affected by prior exonuclease I treatment. In the presence of 0.5 µg of human DNA, treatment of a sample containing 1000 M. tb genome molecules with 10 or 25 units of exonuclease I produces SDA signals of 509000 and 1000000 cpm compared to a single of 700000 cpm obtained without exonuclease.

EXAMPLE 4

Decontamination of Amplicons with Exonuclease VII

[0035] The experiments described in Examples 2 and 3 above are carried out in essentially the same manner as described therein, except exonuclease VII is substituted for exonuclease I as the single strand-specific exonuclease. Exonuclease VII is found to be approximately equally effective to exonuclease I.

EXAMPLE 5

Decontamination of Amplicons with the 3'-5' Exonuclease of T4 DNA Polymerase

[0036] The experiments described in Examples 2 and 3 above are carried out in essentially the same manner as

thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Exonuclease Decontamination Method

(iii) NUMBER OF SEQUENCES: 5

(iv)COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCTGAACCG
GAT
13

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGTTATCCAC
CATAC
15

Claims

1. An amplicon decontamination method comprising adding a single-strand specific exonuclease to a preparation of nucleic acids which have been rendered single-stranded, and reacting the exonuclease with the nucleic acids under conditions suitable for digestion of the nucleic acids such that the amplicons will not serve as a substrate for nucleic acid amplification.

2. A method of Claim 1 wherein the nucleic acid amplification is selected from the group consisting of polymerase chain reaction, ligase chain reaction, strand displacement amplification, transcription based amplification, self-sustained sequence replication, Qβ replicase amplification, nucleic acid sequence-based amplification, repair chain amplification, and boomerang DNA amplification.

3. A method according to Claim 1 wherein the exonuclease is exonuclease VII.

4. A method according to Claim 1, wherein the exonuclease is exonuclease I.

5. A method according to Claim 1 wherein the exonuclease is a DNA polymerase having 3'-5' exonuclease activity.

6. An amplicon decontamination method comprising adding a single-strand specific exonuclease to a preparation of nucleic acids which have been rendered single-stranded, and reacting the exonuclease with the nucleic acids under conditions appropriate for digestion of the nucleic acids such that the amplicons will not serve as a substrate for Strand Displacement Amplification.

7. A method of Claim 6 wherein the nucleic acid to be amplified is from about 10 base pair to about 200 base pair in length.

8. A method of Claim 7 in which the exonuclease is exonuclease VII.

9. A method of Claim 7 in which the exonuclease is exonuclease I.

7. Procédé selon la revendication 6, dans lequel l'acide nucléique à amplifier a une taille d'environ 10 paires de bases à environ 200 paires de bases.

8. Procédé selon la revendication 7, dans lequel l'exonucléase est l'exonucléase VII.

9. Procédé selon la revendication 7, dans lequel l'exonucléase est l'exonucléase I.

10. Procédé selon la revendication 7, dans lequel l'exonucléase est une ADN-polymérase ayant une activité d'exonucléase 3'-5'.